Electrophoretic and Chemical Studies on the X-Ray Damage of Malate Synthase

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The sulfhydryl enzyme malate synthase was shown to undergo an X-ray induced aggregation and inactivation in solution (Zipper and Durchschlag, Radiat. Environ. Biophys. **18,** 99–121 (1980)). Further evidence for the occurrence of aggregation and inactivation and also of fragmentation and partial unfolding of the enzyme upon X-irradiation was obtained by chemical and electrophoretic studies. Irradiation was carried out in a specially designed microcell, experiments were performed on the microlevel. Under conditions of the experiments the formation of H_2O_2 upon X-irradiation could be proven; therefore the influence of H_2O_2 on the enzyme was investigated too. Though the quantitative results of the damaged enzyme particles are influenced by many disturbing factors, the findings allow clear statements on the nature of the effects under investigation.

1) Both X-irradiation and treatment with H_2O_2 caused a decrease of total and an increase of available sulfhydryl groups of the enzyme and led to a loss of enzymic activity. The presence of dithiothreitol turned out to be able to protect the enzyme against X-ray or H_2O_2 induced inactivation. Moreover, addition of dithiothreitol after X-irradiation or H_2O_2 treatment allowed a considerable repair of enzymic activity.

2) Polyacrylamide gel disc electrophoreses of X-irradiated enzyme solutions, performed in the presence of sodium dodecyl sulfate, showed the occurrence of covalently cross-linked subunits (preferably dimers and trimers) and of various definite fragments. Electrophoreses in the absence of the denaturant indicated the occurrence of enzyme aggregation. The effects were more pronounced with increasing X-ray doses. The electrophoreses also clearly reflected a radioprotection by dithiothreitol against cross-linking, but not against fragmentation. Addition of excess of 2-mercaptoethanol or of dithiothreitol to the X-irradiated enzyme clearly demonstrated that part of the covalent cross-links were disulfide bridges; the aggregates themselves, however, were held together primarily by non-covalent bonds. Blocking of exposed enzyme sulfhydryls by means of Ellman's reagent prevented both covalent cross-linking and enzyme aggregation.

3) Similar electrophoretic patterns as found for the X-irradiated enzyme were obtained for the unirradiated enzyme after treatment with H_2O_2. The similarity of the electropherograms, as well as the reversible diminution of enzymic activity and the loss of sulfhydryls in the presence of H_2O_2, suggest an involvement of H_2O_2 in the radiation damage of the enzyme. It seems plausible that oxidation reactions are responsible for the effects caused by X-irradiation or H_2O_2 treatment.

Introduction

Recently, malate synthase (EC 4.1.3.2), the second enzyme of the glyoxylate bypass, has been isolated from baker's yeast for the first time in an electrophoretically pure form [1, 2] and has been characterized by various physico-chemical techniques [1–9]. The native enzyme was shown to be of oblate shape (axial ratio about 1:1/3) and to have a molecular weight of about 175000–187000. Small conformational changes of the enzyme upon substrate binding were elucidated by several techniques of structural analysis. The enzyme could be split into subunits by various denaturing agents. Polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) showed a single band, corresponding to a subunit molecular weight of about 63000–66000. Molecular weight determinations of the native enzyme and its subunits, electron microscopic investigations as well as binding studies suggest a trimeric quaternary structure [6, 2].

Besides the structural characterization of substrate-free malate synthase and of enzyme-substrate com-

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plexes by means of small-angle X-ray scattering (SAXS) [7–9], this technique has also been used for studying X-ray induced damages of the enzyme in solution [7, 10–13]. In the course of this first application of SAXS for the elucidation of radiation damages of biopolymers it has been found that the enzyme suffers both aggregation and inactivation upon irradiation; the experiments established that there was no direct relation between the extent of aggregation and the loss of enzymic activity. Monitoring aggregating malate synthase *in situ* by SAXS has led to a tentative model for a two-dimensional aggregation process. This model was based on the observed retention of the original thickness factor of the enzyme and on the finding of one, and later on of two cross-section factors. Furthermore, in the course of our SAXS experiments a slight decrease of the radius of gyration of the thickness was registered, a phenomenon which might be due to a partial unfolding and/or degradation of the enzyme particles (*cf.* [13]).

It is well established in the field of radiobiology (*e.g.* [14]) that the radiolysis products of water ($H^{\cdot}$, $OH^{\cdot}$, $HO_2^{\cdot}$, O_2^-, e_{aq}^-, H_2O_2) are mainly responsible for for the radiation effects in solution. Under the conditions of our experiments (aqueous solution, presence of oxygen) $OH^{\cdot}$ and possibly O_2^- and $HO_2^{\cdot}$ radicals and H_2O_2 may influence enzymic activity and aggregation, while $H^{\cdot}$ and e_{aq}^- would react with oxygen (*cf.* [10, 12]).

In proteins, especially amino acids containing sulfur or aromatic rings turned out to be highly radiosensitive (*cf.* [15]). Malate synthase was shown to contain a lot of cys, met, tyr, trp, phe [6, 16]; cys and trp were found to be correlated with substrate binding and/or enzymic activity [5, 6]. X-irradiated cys and met might give rise to the formation of sulfenic acids, disulfides or sulfoxides, and might cause non-covalent or covalent cross-links (*cf.* [10, 15]).

In the course of our SAXS experiments [7, 10–12], several substances, such as the substrates, a substrate analogue, dithiothreitol (DTT), ethanol, have proven to reduce considerably the amount of radiation damages by protecting the enzyme against X-ray induced aggregation and/or inactivation. The action of DTT as a radioprotective substance is well-known (*cf.* [14]); its protective effect might be due to scavenging of $OH^{\cdot}$, reactions with non-radical agents (*e.g.* H_2O_2) produced by irradiation, shielding of sensitive groups by specific or unspecific binding to the enzyme, various repair mechanisms (*e.g.* the repair of sulfenic acid products formed by H_2O_2) etc. A variety of reactions leads to a very complex situation, *e.g.* DTT may react with $OH^{\cdot}$ and O_2^- thereby releasing H_2O_2; this situation becomes even more complex in the simultaneous presence of a sulfhydryl enzyme (*cf.* [12]).

The present paper reports on electrophoretic and chemical studies on X-irradiated and on H_2O_2-treated unirradiated malate synthase. We performed this investigation mainly for the following reasons:

(1) to supplement our previous SAXS and radiobiological work with other studies;
(2) to get further insight into the X-ray induced formation of aggregates and perhaps of fragmentation products (especially the occurrence of fragments was unclear from the SAXS studies);
(3) to study the protective effect of DTT;
(4) to elucidate the role of H_2O_2 in the X-ray damage of the enzyme.

Though the electrophoretic and chemical studies of X-irradiated or H_2O_2-treated, and therefore damaged malate synthase were carried out on a microlevel, and though they cannot be performed with the same accuracy as with the monodisperse unirradiated and untreated enzyme, they convincingly demonstrate the occurrence of aggregates, of various fragments, of partial unfolding of the enzyme or its subunits, and of sulfhydryl and activity changes of the enzyme upon X-irradiation or H_2O_2 treatment. They suggest an involvement of H_2O_2 in the X-ray induced damages, reflect the protective effect of DTT against radiation or H_2O_2 damage, and indicate also a post-irradiation or post-treatment repair by DTT. The results are in good agreement with our previous SAXS and radiobiological work. Preliminary abstracts have appeared [17, 18].

Materials and Methods

Chemicals

DTT was obtained from Calbiochem, Luzern, calibration proteins for SDS-gel-electrophoreses (combithek ®) and Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid); "DTNB") from Boehringer, Mannheim, Coomassie Brilliant blue R-250 from Serva, Heidelberg. All other reagents were of A-

grade purity, preferably from Serva, Heidelberg, or Merck, Darmstadt. Quartz-bidistilled water was used throughout.

Enzyme solutions

Malate synthase was isolated from baker's yeast as described elsewhere [8, 2]. For experiments a 5 mM Tris/HCl buffer, pH 8.1, containing 10 mM $MgCl_2$, 1 mM MgK_2EDTA and 0.2 mM DTT, was used as a standard buffer. Stock solutions of the enzyme were prepared by dialysis at 2 °C for 48 h against standard buffer. Enzyme concentrations were determined spectrophotometrically using $A_{280\,nm}^{0.1\%,1cm} = 1.14$.

Treated mixtures

To study the influence of DTT, DTNB and/or H_2O_2 on the enzyme, freshly prepared concentrated solutions of these substances were added to the enzyme solution. DTT was dissolved in standard buffer, DTNB in 0.1 M sodium phosphate buffer, pH 8.0; H_2O_2 was diluted with ice-cold water. The concentrations of these solutions were by a factor of about 10 higher than in the treated mixtures; addition was performed by means of Eppendorf micropipettes or Drummond microcaps®. Incubation with DTT or DTNB was carried out for at least 10 min, incubation with H_2O_2 for about 12 h, at about 0–4 °C.

Irradiation experiments

The X-ray source was a water-cooled tube with copper target and focus of 2×12 mm² (Philips PW 2253/11) that was driven by a Philips PW 1140 power supply at 50 kV and 10 mA. Enzyme solutions of concentration $c = 5$ mg/ml were irradiated in a specially designed microcell which was mounted to the X-ray tube housing on the narrow side of the line-shaped focus. The main advantage of this irradiation cell is the very small volume (50 µl) which is some orders of magnitude smaller than conventionally used in radiobiology (*e.g.* 10 ml, *cf.* [19]).

The irradiation cell (Fig. 1) consisted of a metal holder with a circular opening of 7 mm diameter. The opening was sealed with a mylar foil of 100 µm thickness. A teflon ring was pressed onto the mylar foil. 50 µl of solution were filled into the central opening of the teflon ring and were covered with a sheet of parafilm. This sheet was pressed onto the teflon ring by means of a thrust collar. Thus the solution never came into direct contact with the metal. The cell was thermostated by means of a water circuit to 1–5 °C. The samples were irradiated through the mylar foil by the unfiltered radiation; the dose rate was about 25 krd/min as determined by means of a Fricke dosimeter [20]; in general enzyme samples were irradiated with X-ray doses of 0.1, 0.2, or 1 Mrd. The irradiated samples were removed from the cell after perforating the parafilm.

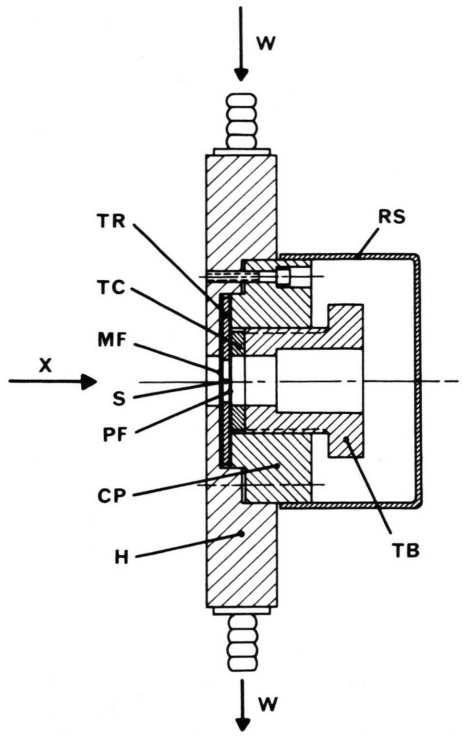

Fig. 1. Schematic drawing of the irradiation microcell. X, X-ray beam; H, holder; MF, mylar foil; TR, teflon ring; S, sample; PF, parafilm; TC, thrust collar; CP, removable connecting piece; TB, thrust bolt; W, water circuit; RS, radiation shield.

For the determination of the H_2O_2 content in various irradiated solutions another type of irradiation cell ($V = 1.25$ ml) was used [10]; this cell was also thermostated by a water circuit. The dose rate amounted to 6.5 krd/min with this cell; the applied voltage was 50 kV, the current was 30 mA.

Determination of hydrogen peroxide

The H_2O_2 content in irradiated solutions was determined by using the well-known reaction with $TiOSO_4$, measuring the absorbance at 410 nm in 1 cm cuvettes and using $\varepsilon = 720\ M^{-1}\,cm^{-1}$ [21]. The

absorption coefficient was checked by means of aqueous H_2O_2 solutions, the content of which was determined by titration with 0.01 N $KMnO_4$.

Determination of sulfhydryl groups

The number of sulfhydryl groups of the enzyme before and after irradiation or H_2O_2-treatment was determined using DTNB [22]. Determination of available sulfhydryls was carried out in 0.08 M sodium phosphate buffer, pH 8.0, containing 1.35 mM Na_2H_2EDTA and no or 1.2 M urea, determination of total sulfhydryls in the same buffers additionally containing 2% SDS. Urea was added to the assay in order to suppress interference by sulfenic acid products (*cf.* [23]). Enzyme solutions were investigated immediately after X-irradiation or 12 h after H_2O_2-treatment. 10–50 µl of the enzyme solutions (5 mg/ml) were brought into the 4 types of buffer; reaction was started by addition of 50 µl 10 mM DTNB. In general, after 2, 15, and 30 min at room temperature, absorbance was measured in 1 cm halfmicro cuvettes (final volume 1.3 ml) at 412 nm. Measurements were performed against blanks containing the same assay except the enzyme; thereby the noxious effect of 0.2 mM DTT inherent in standard buffer could be eliminated.

Enzymic assay

For the enzymic assay the rate of cleavage of the thioester bond of acetyl-CoA was measured directly at 232 nm [24]. The test was performed in a 0.5 cm quartz cuvette (final volume 1.5 ml) in pyrophosphate buffer [25, 2] at 20 °C in Zeiss PMQ II or DMR 10 spectrophotometers. In general 1 µl of the enzyme solution (5 mg/ml) was used in the test; addition was performed by means of a 1 µl Drummond microcap®. The concentration of the enzyme in the assay amounted to 3.3 µg/ml; such a high concentration was used in the test to avoid interference with a concentration dependent dissociation of the enzyme (*cf.* [5]). The specific activities for different samples of the native unirradiated enzyme were determined to be 30–50 U/mg.

The enzymic assay of the treated mixtures was performed in a similar manner. Aliquots (1 µl) of the treated mixtures (10–12 µl) were brought into the assay: as a consequence of the enormous dilution in the assay (factor 1500) a noxious influence of the additives on the enzymic reaction may be excluded largely.

Polyacrylamide gel disc electrophoreses

PAGEs in the presence of the anionic detergent SDS were carried out as described by Weber and Osborn [26], using 5% gels and 0.1 M sodium phosphate buffer pH 7.0, containing 0.1% SDS, as electrophoresis buffer. Proteins for the electrophoresis were incubated at 37 °C for 2 h in 0.01 M sodium phosphate buffer pH 7.0, containing 1% SDS and 1% or no 2-mercaptoethanol (2-ME).

PAGEs in the absence of denaturing agents (and in general in the absence of 2-ME) were performed according to Ornstein [27] and Davis [28], using 5% gels and 0.1 M Tris/glycine buffer pH 8.3 as electrophoresis buffer.

50 µg of malate synthase were applied to both kinds of gels. This corresponds to the normally used amount of protein for gel electrophoreses in the absence of SDS, but is by a factor of about 10 higher as used in conventional SDS-electrophoreses. This mode of acting in the SDS-electrophoreses turned out to be necessary to register all the bands due to subunit fragmentation and/or aggregation, which are relatively weak as compared to the main band (corresponding to the intact subunit of the enzyme).

Electrophoreses in the absence of denaturants were performed at 4 °C for 2 h at 4 mA/tube; SDS-electrophoreses were run at room temperature for 5 h at 5 mA/tube, using bromophenol blue as a tracking dye, and glycerol to increase the viscosity. Electrophoreses were carried out in Shandon electrophoresis apparatus.

After electrophoreses gels were stained by Coomassie Brilliant blue R-250 and destained as described in the literature. In order to make quantitative protein estimations of the SDS-PAGEs, gels were scanned at 560 nm in a Gilford 2400 spectrophotometer. Areas under the peaks were split into Gauss curves, if necessary, and were determined by Simpson integration or were measured planimetrically. Standard curves for colour yields vs. quantity of protein showed a deviation from linearity for > 10 µg protein; estimates for these high amounts of protein were taken from a calibration curve. The PAGEs in the absence of SDS did not deliver reliable quantitative results; the bands above the main band sometimes resembled elongate-shaped clouds instead of pronounced disks, probably due to the anisometric shape of the aggregates which forced an orientation during electrophoresis.

Molecular weights were estimated from SDS-electrophoreses using calibration proteins with polypeptide molecular weights in the range $20\,000 < M_r < 200\,000$. Electropherograms were evaluated by plotting the logarithms of the subunit molecular weights of the calibration proteins vs. migration paths of the single bands of the proteins. Unknown molecular weights could be read from the nearly straight calibration line.

It is clear *a priori*, that quantitative evaluations of the electrophoretic data (M_r and percentage of species) are influenced by many factors: too high intensity of the main band relatively to all other bands including marker bands, different staining intensity of the bands with different sets of experiments, deviations of the calibration curves from linearity, existence of diffuse bands and sometimes of a strong background, different migration paths on different gels (*e. g.* caused by different homogeneity and swelling of gels) and under different conditions (*e. g.* smaller migration paths in the presence of 2-ME), different stability of the enzyme against X-irradiation and therefore slightly different patterns (*e. g.* due to different ages of enzyme samples used and/or preceding small proteolytic effects), turbidity of the solutions after storage of the enzyme irradiated with high X-ray doses or after treatment with large amounts of H_2O_2, etc.

For the sake of clarity, patterns were standardized with respect to the position of the main band. The intensity of the bands was indicated in the figures by different hatching in order to allow at least semi-quantitative estimations.

Results and Discussion

Before performing electrophoretic studies under several conditions, we undertook the task to verify the assumption of a H_2O_2 production during X-irradiation, and of a radiation induced change of sulfhydryl groups and of activity of the enzyme.

1. Evidence for H_2O_2 formation and for sulfhydryl and activity changes upon X-irradiation of enzyme solutions

Determination of H_2O_2

The formation of H_2O_2 during X-irradiation of aqueous solutions was checked by determination of the H_2O_2 content of various irradiated solutions. The results (*cf.* Table I) clearly reflect the formation of H_2O_2 upon X-irradiation.

The concentration of H_2O_2 in irradiated pure water was found to be 0.19 mM for a dose of 0.2 Mrd. In general, X-irradiation of various aqueous solutions containing the enzyme and/or the buffer components led to a lower H_2O_2 content. In a similar way, in X-irradiated aqueous solutions of the substrates a reduced concentration of H_2O_2 has been reported [12].

In the present study the lowest H_2O_2 concentration in irradiated buffer was registered for buffer without DTT. This behaviour might be due to the presence of Tris/HCl and MgK_2EDTA, as follows from a comparison of the values found for these substances dissolved in pure water.

Increasing amounts of DTT in the buffer caused an increasing enlargement of the H_2O_2 content in the buffer. The presence of DTT in pure water led to a different behaviour, namely at low concentrations of DTT (0.2 mM) to a considerable diminution of the H_2O_2 content, as compared to pure water, and at high concentrations of DTT (2 mM) to an enhanced concentration of H_2O_2.

The presence of the sulfhydryl enzyme malate synthase in buffer led, similar to the action of DTT in buffer, to an enlargement of the H_2O_2 concentra-

Table I. Formation of H_2O_2 upon X-irradiation (D = 0.2 Mrd) of various aqueous solutions.

Solution	H_2O_2 content [mM] [a]
H_2O	0.19
5 mM Tris/HCl pH 8.1 in H_2O	0.08_5
10 mM $MgCl_2$ in H_2O	0.19
1 mM MgK_2EDTA in H_2O	0.11
0.2 mM DTT in H_2O	0.13
2 mM DTT in H_2O	0.25
buffer [b] (no DTT)	0.08
buffer [b] (0.2 mM DTT)	0.10_5 [c]
buffer [b] (2 mM DTT)	0.13
enzyme [d] in buffer [b] (no DTT)	0.10
enzyme [d] in buffer [b] (0.2 mM DTT)	0.13
enzyme [d] in buffer [b] (2 mM DTT)	0.15

[a] Accuracy: ± 4%.
[b] Buffer: 5 mM Tris/HCl pH 8.1, 10 mM $MgCl_2$, 1 mM MgK_2EDTA, and 0 or 0.2 or 2 mM DTT. Buffer containing 0.2 mM DTT was used as a standard buffer (*cf.* Materials and Methods).
[c] This value was found for fresh standard buffer; after some time of storage the value decreased to the value measured for buffer containing no DTT.
[d] Enzyme: dialyzed against the corresponding buffer, c = 0.5 mg/ml.

tion in the buffer. This effect was more pronounced in the simultaneous presence of DTT.

As already mentioned (*cf.* [12]), DTT is able to protect the enzyme against radiation damage, probably by various mechanisms (including the repair of sulfenic acid products formed by H_2O_2). The protective effect of DTT against aggregation was provided by reduced DTT and to a certain extent also by DTT in the oxidized form, in contrast to inactivation where oxidized DTT showed no protective effect. The oxidation of DTT during irradiation could be established by measurement of the UV absorption spectrum of aqueous solutions of DTT and the increase of absorbance at 283 nm (*cf.* the spectra of reduced and oxidized DTT [29]).

Determination of sulfhydryl groups

Because of the well-known sensitivity of sulfhydryl groups, it was of particular interest to compare the number of sulfhydryl groups of unirradiated and of X-irradiated or H_2O_2-treated malate synthase, both in the absence or presence of denaturing agents (*cf.* [10]). The results from these experiments are presented in Table II.

For the unirradiated untreated enzyme 3.1 ± 0.2 out of 17.2 ± 1 sulfhydryl groups were found to be accessible towards Ellman's reagent in the native state. These values are in good agreement with the values of 3.6 ± 0.2 and 17 ± 1 reported earlier [6]. Full colour development was obtained after about 5 min in the determination of available sulfhydryls. In the case of total sulfhydryls full colour was reached after about 15 min, after completion of unfolding by SDS.

Upon X-irradiation with a dose of 1 Mrd a decrease of readily available sulfhydryl groups could be observed when the measurement was performed shortly after addition of DTNB to the enzyme solution. After some time, however, a significant increase of available sulfhydryls could be registered, probably due to a partial unfolding of the enzyme. Colour development was complete after 30 min. The simultaneous presence of 1.2 M urea in the assay led to slightly higher values at the short incubation times, obviously due to a promotion of the unfolding of the X-ray damaged enzyme, but caused only slight changes of the values after full colour development.

Determination of the total number of sulfhydryls of the X-irradiated enzyme established a loss of sulfhydryls corresponding to about 1.4 sulfhydryls per enzyme molecule, independent of the presence or absence of 1.2 M urea in the assay.

The increase of available sulfhydryls and the decrease of total sulfhydryls of X-irradiated malate synthase is in accord with the results found for irradiated lactate dehydrogenase [30, 31].

The sulfhydryls of the unirradiated H_2O_2-treated enzyme (treated with 33 mM H_2O_2) were found to be changed in an analogous way as the sulfhydryls of the X-irradiated enzyme, however, the increase of available sulfhydryls was not so pronounced. This probably indicates that the structure of the H_2O_2-

Table II. Determination of sulfhydryl groups of malate synthase[a] after X-irradiation or treatment with H_2O_2.

X-irradiation *D* [Mrd]	Treatment H_2O_2 [mM]	Time [min] after addition of DTNB	Number of sulfhydryls[b] per enzyme molecule			
			available		total	
			no urea	urea	no urea	urea
–	–	≦ 2	2.3–2.6	2.9–3.0	≦ 14.8	≦ 14.8
		15	3.1	3.1	17.1	17.2
		30	3.1	3.6[c]	17.6	17.0
1	–	≦ 2	1.0–6.2	3.8–7.3	≦ 13.1	≦ 12.0
		15	8.6	11.1	14.8	15.5
		30	10.1	10.7	15.8	15.8
–	33	≦ 2	1.2–2.1	1.4–2.6	≦ 13.9	≦ 13.0
		15	4.4	5.4	14.4	15.2
		30	4.6	6.3[c]	14.6	15.2

[a] Enzyme: dialyzed against standard buffer, $c = 5$ mg/ml.
[b] Accuracy: ± 5–10%.
[c] Further increase upon storage of the assay, probably due to increasing unfolding of the enzyme in the presence of 1.2 M urea.

Table III. Inactivation of malate synthase[a] upon X-irradiation or after treatment with H_2O_2 and/or DTNB, and protection and repair of the enzyme by DTT.

Inactivation and protection					Repair	
DTT initial concentration [mM]	X-irradiation D [Mrd]	Treatment DTNB [mM]	Treatment H_2O_2 [mM]	Activity[b,c] (% of initial activity)	DTT added after X-irradiation or treatment [mM]	Activity[b,d] (% of initial activity)
0.2, 2, 4, or 10	–	–	–	100		
0.2	0.1	–	–	52		
0.2	0.2	–	–	34		
0.2	1	–	–	3	2 or 10	9
2	1	–	–	22	2	44
				3[e]	2	31
0.2	–	–	0.33	100		
0.2	–	–	3.3	78	2	100
0.2	–	–	33	33[f]	2 or 10	61
0.2	–	–	330	0	2, 10, or 100	0
2	–	–	3.3	96	2	100
2	–	–	33	20[f]	2 or 10	45
2	–	–	330	0	2, 10, or 100	0
0.2	–	5	–	0		
2	–	5	–	3		
0.2 or 2	–	5	3.3[g]	0		

[a] Enzyme: dialyzed against standard buffer, $c = 5$ mg/ml, initial specific activity $\approx 40-50$ U/mg.
[b] Accuracy: $\pm$ 5%.
[c] Measurement of enzymic activity shortly after X-irradiation or 12 h after treatment, except the value designated with annotation e.
[d] Measurement of enzymic activity 6 h after addition of DTT.
[e] Measurement of enzymic activity 17 h after X-irradiation.
[f] The absolute values differ by about $\pm$ 20%, this is probably due to the experimental handling of the H_2O_2-treated enzyme: local excess of H_2O_2 (1 µl) and some coagulation of the enzyme as a consequence of H_2O_2 addition to the enzyme (10 µl) etc.
[g] H_2O_2 was added to the DTNB-treated enzyme.

treated enzyme is less damaged and opened than the X-irradiated enzyme. The absence of urea in the assay yielded slightly lower estimates for the total sulfhydryls, probably due to disturbing interference with sulfenic acid products.

Determination of enzymic activity

In the previous studies [10, 12] enzymic activity of malate synthase was found to decrease about exponentially with increasing X-ray dose; in the presence of 0.2 mM DTT a G-value* for the inactivation of 0.099 heV^{-1} was found; the addition of DTT turned out to have a remarkable influence on the rate of inactivation.

* The G-value gives the number of molecules which will be inactivated per 100 eV of absorbed radiation energy (*cf.* [32]).

In the present study enzymic activity was investigated before and after X-irradiation or treatment with H_2O_2 and/or DTNB in the presence of different concentrations of DTT. DTT was added to the enzyme solution before and/or after X-irradiation or treatment. Results are documented in Table III.

Addition of DTT to unirradiated untreated enzyme did not result in a change of activity within 24 h after addition. This indicates an enzyme with intact essential sulfhydryl groups.

X-irradiation of the enzyme samples in the presence of 0.2 mM DTT with doses as used later on for the electrophoreses yielded considerable losses of enzymic activity; the losses observed at high X-ray doses were definitely smaller than expected for a strictly exponential decay of activity. Post-irradiation treatment of the enzyme solutions with 2 or 10 mM DTT caused a considerable restoration of activity.

X-irradiation of the enzyme samples in the presence of 2 mM DTT yielded an essentially smaller loss of activity, when activity measurements were carried out shortly after irradiation. This behaviour reflects protection of the enzyme by DTT. Post-irradiation treatment of the enzyme solutions with 2 mM DTT again led to a considerable repair. A similar restoration of enzymic activity by DTT, as found for malate synthase, has been observed in the case of irradiated glyceraldehyde-3-phosphate dehydrogenase; this restoration has been attributed to a sulfenic acid repair [33]. It seems plausible that in the case of malate synthase the repair phenomenon might also be due to the reversible formation of sulfenic acid products.

When activity measurements were performed 17 h after irradiation, the enzyme irradiated in the presence of 2 mM DTT showed the same low activity as found for the enzyme irradiated with the same X-ray dose (1 Mrd) in the presence of 0.2 mM DTT. The repair of this stored sample by treatment with 2 mM DTT was lower than found with the unstored sample, but essentially higher than with the sample irradiated in the presence of 0.2 mM DTT. The further decrease of activity upon post-irradiation storage may be due to secondary reactions which follow the preceding X-irradiation and obviously lead to irreversibly damaged products (*cf.* [10–12]). These reactions may include unfolding and/or aggregation, as well as further oxidation reactions of the enzyme. For instance, dissolved oxygen or excess of H_2O_2 may further oxidize the initial sulfenic acids to the sulfonic acid state [34].

The inactivation of the unirradiated enzyme (0.2 mM DTT) after treatment with different amounts of H_2O_2 was also confirmed. The presence of 3.3 or 33 mM H_2O_2 in the enzyme solutions resulted in considerable losses of enzymic activity; the presence of 330 mM H_2O_2 caused a complete loss of activity. Addition of 2 or 10 mM DTT to the H_2O_2-treated enzyme again yielded a restoration of activity, except the enzyme treated with 330 mM H_2O_2; the repair was complete for the enzyme sample treated with 3.3 mM H_2O_2.

Treatment of the enzyme with 3.3 mM H_2O_2 in the presence of 2 mM DTT led to a minor decrease of activity, which was also completely reversible by addition of 2 mM DTT after treatment. For the enzyme treated with 33 mM H_2O_2 the decrease of activity seems to be pronounced in the presence of 2 mM DTT, as compared to the enzyme in the presence of 0.2 mM DTT. Unfortunately, the experimental conditions do not allow far-reaching statements in this case.

The addition of large amounts of H_2O_2 (> 33 mM) to the unirradiated enzyme led to a visible turbidity of the solution, obviously due to a distinct aggregation. This formation of aggregates was also reflected by a considerable increase of viscosity.

The addition of 5 mM DTNB to the enzyme (0.2 mM DTT) resulted in a total loss of enzymic activity. Obviously the enzyme was modified by DTNB in such a manner (*cf.* [22]) to prevent enzymic reaction. But this, of course, does not necessarily mean that the sulfhydryl groups are located directly in the active centre (*cf.* [35]). The simultaneous presence of DTNB and 2 mM DTT led to a small rest of activity. The simultaneous presence of DTNB and H_2O_2 resulted in a complete loss of activity, even in the presence of 2 mM DTT.

2. Electrophoreses

Experiments were carried out with malate synthase in standard buffer containing 0.2 mM DTT (*cf.* Materials and Methods), and with enzyme solutions to which 2 mM freshly dissolved DTT had been added, in order to study the protective effect of DTT against X-irradiation or other treatments. Electrophoreses were performed with unirradiated and untreated enzyme samples, and with samples which had been X-irradiated, or treated with H_2O_2, or treated with DTNB, or treated with DTNB and afterwards X-irradiated or H_2O_2-treated. PAGEs of the enzyme solutions were performed immediately after irradiation or DTNB-treatment or 12 h after H_2O_2-treatment.

PAGEs of these irradiated and/or treated enzyme solutions were performed in the presence or absence of SDS, to show the existence or formation of aggregates and/or fragments of the enzyme or its subunits. While the PAGEs in SDS allow clear statements concerning the particle weight of subunits or covalently linked subunits or fragments thereof, the patterns in the absence of SDS are influenced by the particle weight and the shape and charge of the enzyme or its aggregates or fragments.

For the PAGEs in SDS, enzymes were incubated in the presence or absence of 2-ME. Experiments in the absence of 2-ME should prove the existence or formation of all possible covalent linkages, experi-

ments in its presence (as usually done) were performed in order to achieve disruption of disulfide bonds. The differences in the electrophoretic patterns may be attributed to the existence of disulfides.

PAGEs in the absence of SDS or any other denaturing agent were performed, as usually done, without addition of 2-ME. In order to be able to correlate the both types of electrophoreses with respect to reducing agents, we additionally performed some of these electrophoreses in the presence of 2-ME or DTT, 2 h after addition.

Electrophoreses of unirradiated and untreated enzyme

PAGEs of unirradiated and untreated enzyme in the presence or absence of SDS (*cf.* Figs. 2, 3, 6–8: 0 Mrd, 0 mM H_2O_2, or 0 mM DTNB) yielded a single band, indicating an electrophoretically pure enzyme built up of identical or very similar subunits. This statement holds both in the presence or absence of 2-ME or DTT. Both in the native and in the denaturated state of the enzyme there is no indication for the existence of interchain disulfide bridges, or of other multiples of the subunit or of the enzyme, or of fragmentation products. These findings also rule out the formation of such products during the performance of the electrophoreses.

Electrophoreses of X-irradiated enzyme

a) PAGEs in SDS

PAGEs in SDS, performed in the presence or absence of 2-ME (Figs. 2–3), allow statements concerning several changes of the enzyme subunit upon X-irradiation:

As can be seen from Figs. 2a and 3a, the SDS-PAGEs of the enzyme irradiated in the presence of 0.2 mM DTT show, besides the strong band of the subunit of the enzyme, several bands corresponding to particles of lower or higher molecular weight than the subunit. The bands below the main band obviously reflect definite fragmentation products. With increasing time of irradiation, *i.e.* with increasing X-ray doses, the intensity of the main band decreases, the number of bands below the main band increases, and the bands become more diffuse, especially in the absence of 2-ME. The occurrence of one or two weak bands (or sometimes more, very weak bands) above the main band indicates small amounts of particles with about the double or threefold (or *n*-fold) molecular weight of the subunit, as can be estimated from their migration paths (*cf.* Fig. 4). The number and intensity of these bands is reduced in the presence of 2-ME, but weak bands corresponding to the double subunit molecular weight (and sometimes a second band corresponding to the threefold molecular weight) are still present at higher X-ray doses. At high X-ray doses, where

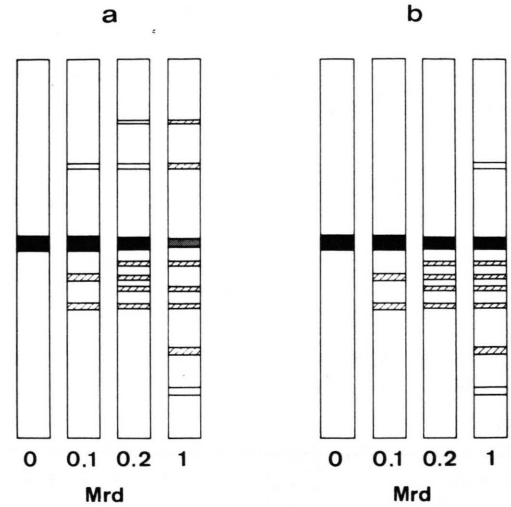

Fig. 2. PAGEs of unirradiated and X-irradiated malate synthase in the presence of SDS, but without 2-ME. The samples (enzyme concentration $c = 5$ mg/ml) were irradiated with doses of 0, 0.1, 0.2 or 1 Mrd in the presence of 0.2 mM DTT (a) or 0.2 mM DTT + 2 mM freshly dissolved DTT (b).

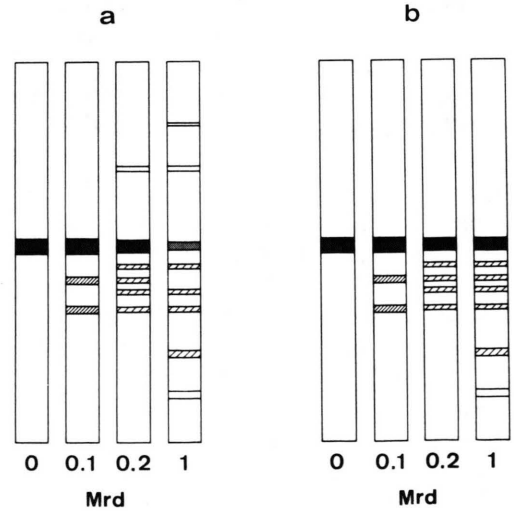

Fig. 3. PAGEs of unirradiated and X-irradiated malate synthase in the presence of SDS and 2-ME. Samples and irradiation conditions were the same as described in the legend of Fig. 2.

considerable fragmentation and unfolding occur, the bands above the main band are accompanied by additional very weak diffuse bands of lower molecular weight, probably due to multiples of fragments, and also by a strong background (*cf. e. g.* Fig. 5a, b).

Electrophoretic patterns of the enzyme irradiated in the presence of 2 mM freshly dissolved DTT are shown in Figs. 2b and 3b. They strongly resemble the patterns in the presence of the lower content of DTT (Figs. 2a and 3a), apart from the following differences: the decrease of the intensity of the main band is less pronounced, and even upon prolonged irradiation the main band is relatively sharp; the number of bands below the main band differs slightly (and these bands are also less diffuse); in the presence of 2-ME there is no band above the main band, even upon prolonged irradiation, while in the absence of 2-ME a weak band occurs at the highest X-ray dose used. The retention of intensity and sharpness of the main band of the SDS-PAGEs in the presence of 2 mM DTT during X-irradiation (*cf.* Figs. 2a with 2b, and 3a with 3b: 1 Mrd) clearly shows a protection of the enzyme subunit by DTT.

In the SDS-PAGEs the formation of covalent cross-links in irradiated malate synthase is obviously indicated by the occurrence of the bands corresponding to multiples of the subunit molecular weight. This phenomenon is most pronounced at the highest X-ray dose used and at low concentrations of DTT during X-irradiation and in the absence of 2-ME during electrophoresis (Fig. 2a: 1 Mrd), and is quantitatively suppressed in the presence of high DTT concentrations during X-irradiation and in the presence of 2-ME during electrophoresis (Fig. 3b: 1 Mrd).

Though both 2-ME and DTT may suppress covalent cross-linking in the PAGEs and both substances are known to be able to reduce disulfides, their action in this case must certainly be a different one. While DTT may act primarily as a radioprotector by means of several mechanisms during X-irradiation (and to a certain extent afterwards by a post-irradiation repair, if not all DTT has been used up), the role of 2-ME, added together with SDS after irradiation, is obviously only that of a reducing agent which breaks the disulfide linkages already formed upon irradiation.

The fact that the intensity of the bands above the main band of the irradiated enzyme decreases significantly and sometimes bands disappear completely upon addition of 2-ME – especially of the samples irradiated with low X-ray doses – (*cf.* Figs. 2a with 3a, and 2b with 3b), suggests that disulfide bridges play an essential role for covalent cross-linking, and that their formation may already be prevented during X-irradiation when enough DTT is present (*cf.* Figs. 2a with 2b, and 3a with 3b).

The fact that at low DTT concentration during X-irradiation and even in the presence of 2-ME during electrophoresis (Fig. 3a: 0.2 and 1 Mrd) weak bands, corresponding to the double or threefold subunit molecular weight, appear at high X-ray doses are hints for additional covalent cross-links other than disulfide bonds.

The presence of 2 mM DTT is obviously capable of preventing the formation of both disulfides and other covalent cross-links (Fig. 3b: 0.2 and 1 Mrd). The weak band at high DTT concentration and in the absence of 2-ME at the highest X-ray dose used (Fig. 2b: 1 Mrd) may be explained by a disulfide bond, the formation of which could not be prevented by 2 mM DTT (which obviously has been used up upon prolonged irradiation); this bond, however, disappeared after addition of excess of 2-ME (Fig. 3b: 1 Mrd).

In contrary to the bands above the main band, the intensity of the bands below it is not significantly influenced by the presence of DTT during X-irradiation or by the subsequent addition of 2-ME during SDS-electrophoresis. That means that the presence of DTT during X-irradiation does not prevent fragment formation (indeed the number of fragment bands increased slightly). The major part of fragments of malate synthase cannot be linked by disulfide linkages, as follows from the similar electrophoreses in the absence or presence of 2-ME (*cf.* Fig. 2a with 3a). An opposite behaviour has been reported for the electropherograms of irradiated lactate dehydrogenase, where enzyme fragments became obvious only after reduction [19].

b) Determination of molecular weights of subunit aggregates and fragments

A quantitative determination of the molecular weights of subunit aggregates or fragments was carried out using their migration distances in the gels obtained from SDS-PAGEs in the presence of marker proteins. After having attributed molecular weights to all possible bands, the following values for M_r (all

with an accuracy of about ± 5–10%) were obtained (Fig. 4): 65000 for the main band (in accordance with previous findings [6, 2]); 130000 and 195000 for the bands above the main band; 54000, 49000, 44000, 38000, 27000, 20000, and 14000 for the bands below the main band. *E.g.* X-irradiation of the enzyme (in standard buffer) with a dose of 1 Mrd yields in the SDS-PAGEs without 2-ME all the particles mentioned above except that of 49000 (*cf.* Fig. 2a: 1 Mrd and Fig. 5a with Fig. 4). A rough estimation of the intensity of the bands, obtained after scanning the stained gels spectrophotometrically, yields for this example (Fig. 5a) the following percentages (all values with an accuracy of about ± 20%): 42% monomer, 11% dimer, 6% trimer, and 41% fragments. Addition of 2-ME (Fig. 5b) results in a pronounced decrease of the intensity of the dimer (8%) and trimer (4%), but does not cause a significant change of the intensities of the fragments (38%). An exact determination of the percentages can hardly be performed at the moment because of the limited reproduction of quantitative results of the electrophoreses (*cf.* Materials and Methods).

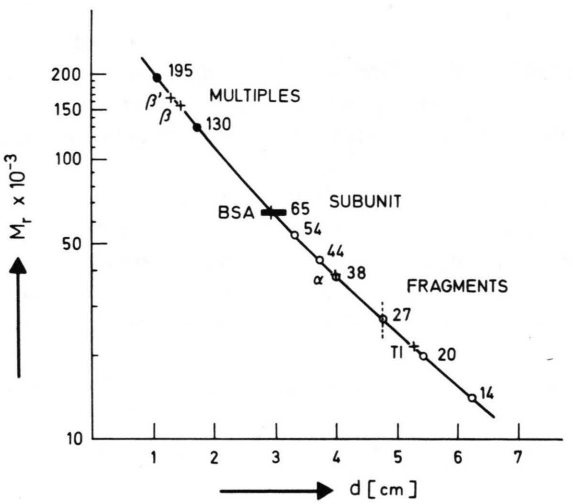

Fig. 4. Semi-log plot of polypeptide chain molecular weights M_r of standard marker proteins and of malate synthase molecules against distance d of migration, as obtained by PAGEs in the presence of SDS. For this plot an X-irradiated enzyme ($c = 5$ mg/ml, 0.2 mM DTT, $D =$ 1 Mrd; PAGE without 2-ME; *cf.* Fig. 2a: 1 Mrd) was used. (▬), enzyme subunit; (●), multiples of the enzyme subunit; (○), fragments of the enzyme subunit; (+), marker subunits; (), bromophenol blue. The marker proteins used were trypsin inhibitor (TI) from soybean, bovine serum albumin (BSA), and the α, β and β′ subunits of RNA-polymerase from *E. coli* (core enzyme); $M_r = 21500$ (TI), 39000 (α), 66200 (BSA), 155000 (β), 165000 (β′).

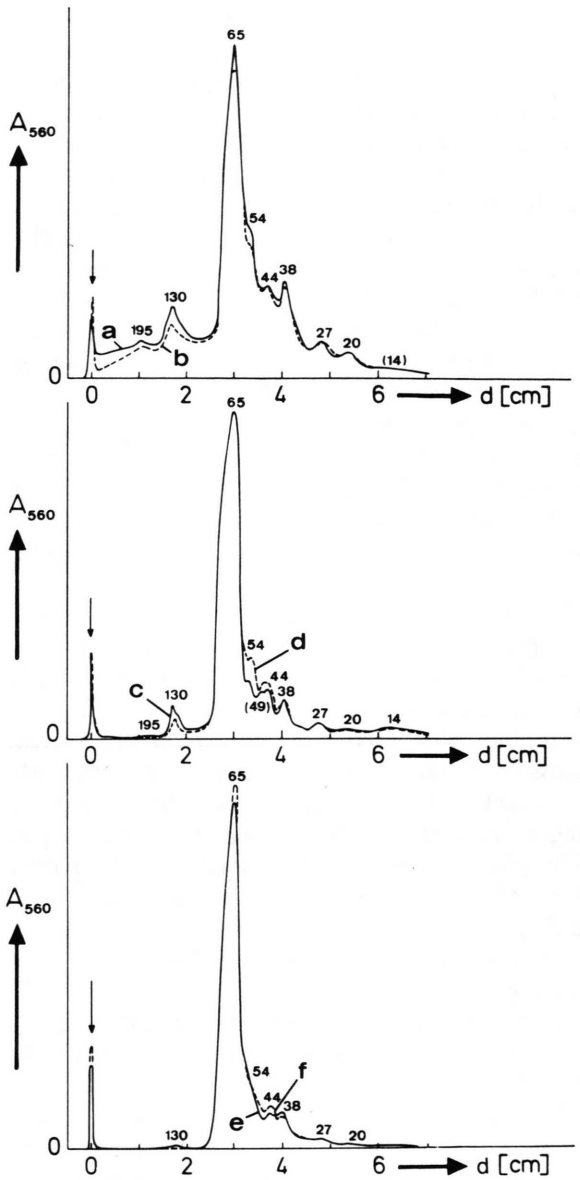

Fig. 5. Densitometer traces of electrophoretic separations of X-irradiated or H_2O_2-treated unirradiated or DTNB-treated and X-irradiated malate synthase, as obtained by PAGEs in the presence of SDS: X-irradiated enzyme, 1 Mrd, PAGE without (a) or with 2-ME (b); H_2O_2-treated enzyme, 330 mM H_2O_2, PAGE without (c) or with 2-ME (d); DTNB-treated and X-irradiated enzyme, 1 Mrd, PAGE without (e) or with 2-ME (f). Begin of the gels is marked by arrows. In order to allow correlation with the electropherograms, molecular weights are given at the top of the peaks (in $M_r \times 10^{-3}$); the band due to $M_r = 14000$ is not shown in Figs. 2 and 3.

Summarizing we may state, that the SDS-PAGEs clearly show the formation of covalent multiples (especially dimers and trimers), as well as the formation of various definite fragments (about 5–10% of each fragment) with molecular weights which differ by about 5000–10000. At low doses the covalent multiples are preferably disulfides, at higher doses a significant amount of other covalent bonds was formed additionally.

c) PAGEs in the absence of denaturing agents

PAGEs in the absence of denaturants (Fig. 6) reflect changes of the whole enzyme:

As Fig. 6a shows, X-irradiation of the enzyme in the presence of 0.2 mM DTT leads to a broadening of the main band, due to the native enzyme, with increasing X-ray dose, indicating a change of the net charge and/or a beginning of unfolding and/or fragmentation. Additionally a second diffuse band occurs above the main band, probably due to the formation of aggregates. Upon prolonged irradiation no defined band can be observed. This behaviour at high X-ray doses may reflect the formation of high-molecular particles, which are unable to enter the pores of the gel. The formation of a broad spectrum of differently charged unfolded products and/or small fragments may lead to a similar pattern.

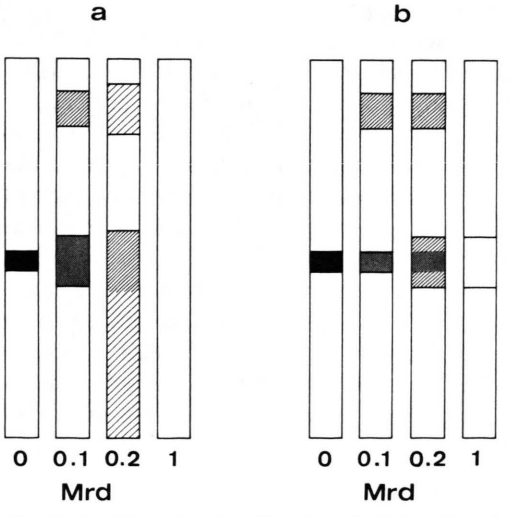

Fig. 6. PAGEs of unirradiated and X-irradiated malate synthase in the absence of SDS and without 2-ME. Samples and irradiation conditions were the same as described in the legend of Fig. 2. Similar electropherograms were obtained after addition of ≦ 10% 2-ME or of additional 2 mM DTT to the X-irradiated enzyme prior to the electrophoresis.

Electrophoretic patterns of the enzyme irradiated in the presence of 2 mM freshly dissolved DTT are given in Fig. 6b. As compared to the patterns at lower content of DTT (Fig. 6a), the main band and the band above the main band broaden to a lesser extent, and the main band remains slightly visible even at the highest X-ray dose used.

The PAGEs in the absence of SDS clearly show a protection of the enzyme by DTT. The less pronounced broadening of the main band in the presence of 2 mM DTT is obviously due only to a protection against a change of the net charge and/or unfolding of the enzyme, because a radioprotective effect of DTT against fragmentation has already been excluded by the SDS-PAGEs.

The PAGEs in the absence of SDS were performed, as usually done, without 2-ME. No significant change of the electropherograms was found, however, when excess of the reducing agents 2-ME or DTT was added after X-irradiation. This indicates that the aggregates formed are not held together by disulfide bridges or at least not exclusively by them.

Electrophoreses of H_2O_2-treated enzyme

PAGEs of H_2O_2-treated unirradiated enzyme (final concentration of H_2O_2 in the treated mixture: 0.33, 3.3, 33 or 330 mM) were performed about 12 h after addition of H_2O_2, in the presence or absence of SDS, and under similar conditions as used for the irradiated enzyme.

PAGEs in SDS in the absence of 2-ME (*cf.* Fig. 7a: 0.33–33 mM H_2O_2) show, with increasing H_2O_2 content, one, or two (or more very weak) bands above the main band, corresponding to multiples of the molecular weight of the subunit, but no bands below the main band corresponding to fragmentation products.

Only at very high concentrations of H_2O_2 (≧ 330 mM) the intensity of the main band decreases significantly and bands below the main band occur in the electropherograms (*cf.* Fig. 7a: 330 mM; Fig. 5c). These bands below the main band, indeed, have the same migration paths as the bands of the X-irradiated samples (*cf. e.g.* Fig. 2a: 1 Mrd). Smaller estimates for the intensities of the bands of the subunit multiples and of the fragments were found, as compared to the bands of the X-irradiated samples: about 86% monomer, 1.9% dimer, 0.35% trimer,

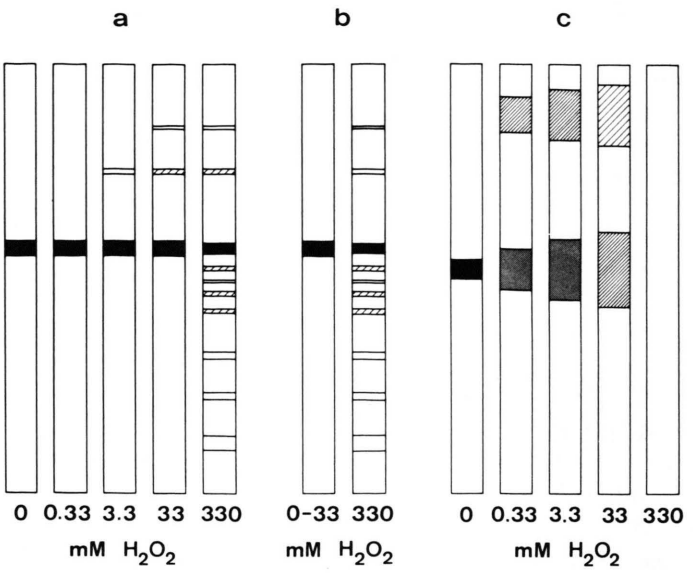

Fig. 7. PAGEs of unirradiated untreated and of H_2O_2-treated unirradiated malate synthase in the presence of SDS and without 2-ME (a), in the presence of SDS and 2-ME (b), or in the absence of SDS and without 2-ME (c). Different amounts of H_2O_2 (final concentration: 0, 0.33, 3.3, 33, 330 mM) were added to enzyme samples (c = 5 mg/ml, 0.2 mM DTT). About 12 h after mixing, samples were incubated with SDS and afterwards electrophoreses were performed. Similar patterns as shown in c were obtained after addition of ≦ 10% 2-ME to the H_2O_2-treated enzyme prior to the electrophoresis.

and 12% fragments for the PAGEs in the absence of 2-ME.

PAGEs in SDS, performed in the presence of 2-ME (Fig. 7b: 0.33–33 mM), only yielded one single band, as observed for the subunit of the untreated unirradiated enzyme. At very high concentrations of H_2O_2 (Fig. 7b: 330 mM; Fig. 5d) one or two weak bands above the main band and several fragments occur in the electropherograms too. Intensity estimates yield about 80% monomer, 1.3% dimer, 0.2% trimer, and 18% fragments for the PAGEs in the presence of 2-ME. In contrary to the fragment bands produced by X-irradiation, the fragment bands of the H_2O_2-treated enzyme were slightly reinforced by 2-ME.

A comparison of the SDS-PAGEs in the absence or presence of 2-ME (*cf.* Figs. 7a with 7b, and 5c with 5d) indicates the formation of covalently linked enzyme subunits, partly disulfides, and of fragments, as a consequence of H_2O_2 treatment.

PAGEs in the absence of denaturants (*cf.* Fig. 7c) show, with increasing H_2O_2 content, a broadening and a decrease of the intensity of the main band and the occurrence of one (or more) bands above the main band, probably due to the formation of aggregates. At a H_2O_2 concentration of ≧ 330 mM all bands disappear, probably due to the formation of high-molecular aggregates, a phenomenon which was also suggested by the turbidity of the enzyme solution and by the increase of viscosity.

When excess of 2-ME was added after H_2O_2 treatment but prior to the electrophoresis, PAGEs in the absence of denaturants yielded similar patterns (not shown) as the PAGEs performed in the absence of 2-ME (Fig. 7c).

The presence of 2 mM DTT (PAGEs not shown) besides large amounts of H_2O_2 (330 mM) is, of course, not able to reduce cross-linking and aggregation significantly.

There is, without doubt, a great analogy between the patterns obtained after X-irradiation or H_2O_2 treatment, both for the PAGEs in the presence or absence of SDS. Excess of H_2O_2 obviously leads to fragments with the same molecular weights as observed in the irradiated enzyme samples. Some of the bands of fragment molecular weights (as well as dimers) have also been obtained after succinylation, after performic acid treatment or for aged unirradiated samples (*cf.* [6, 35], detailed results to be published). This indicates that oxidation processes are obviously responsible for the observed damages (*i.e.* formation of aggregates, unfolding and fragmentation) caused by X-irradiation or H_2O_2 treatment.

Electrophoreses of DTNB-treated enzyme

The PAGEs in SDS of the X-irradiated or H_2O_2-treated unirradiated enzyme clearly showed the formation of a remarkable amount of disulfides. There-

fore some additional experiments were performed in the presence of DTNB to investigate the effects after having blocked the sulfhydryls exposed on the enzyme surface.

PAGEs of DTNB-treated unirradiated, of DTNB-treated and X-irradiated, and of DTNB- and H_2O_2-treated unirradiated enzyme were performed, in the presence or absence of SDS (Fig. 8), and under similar conditions as used previously for the X-irradiated or H_2O_2-treated enzyme.

PAGEs of DTNB-treated unirradiated enzyme (Fig. 8a–d: 5 mM DTNB) showed no significant changes of the main band as compared to the untreated unirradiated enzyme and no bands due to fragmentation. There occurs only a slight broadening of the main band and a weak band due to enzyme aggregation in the PAGEs without SDS and without 2-ME, and a weak band ($\approx 0.3\%$) above the main band in the SDS-PAGEs without 2-ME [6] due to covalent subunit cross-linking. This behaviour is obviously due to small amounts of disulfide bridges between enzyme particles or their subunits, formed by sulfhydryl-disulfide exchange reactions as a consequence of DTNB binding (*cf.* [22]); pH-values above neutrality are known to lead to enhanced sulfhydryl-disulfide exchange reactions (*cf.* [36]). Subsequent addition of 2-ME resulted in a complete loss of the multiple bands in the electropherograms.

Surprisingly, addition of 2 mM DTT to the enzyme before DTNB-treatment (5 mM) leads in the SDS-PAGEs (not shown) to strong dimer and trimer bands (about 6 or 1% respectively) for the PAGEs without 2-ME; the bands vanish nearly completely, however, after addition of 2-ME to the SDS-PAGEs. In a similar way in the PAGEs without SDS and without 2-ME strong bands above the main band occur, which may also be removed with 2-ME.

X-irradiation of DTNB-treated enzyme in the presence of 0.2 mM DTT caused an enhancement of the intensity of the yellow colour of the enzyme-DTNB solution with increasing X-ray dose. This behaviour is in accord with the aforementioned increase of exposed sulfhydryls due to an X-ray induced partial unfolding of the enzyme.

SDS-PAGEs of the DTNB-treated and X-irradiated enzyme (Fig. 8a and b: 5 mM DTNB, 1 Mrd) only showed a weak band above the main band in the PAGEs without 2-ME, as the SDS-PAGEs of the DTNB-treated unirradiated enzyme; additionally several bands below the main band due to fragmentation occurred, both in the absence or presence of 2-ME during electrophoresis. For a dose of 1 Mrd

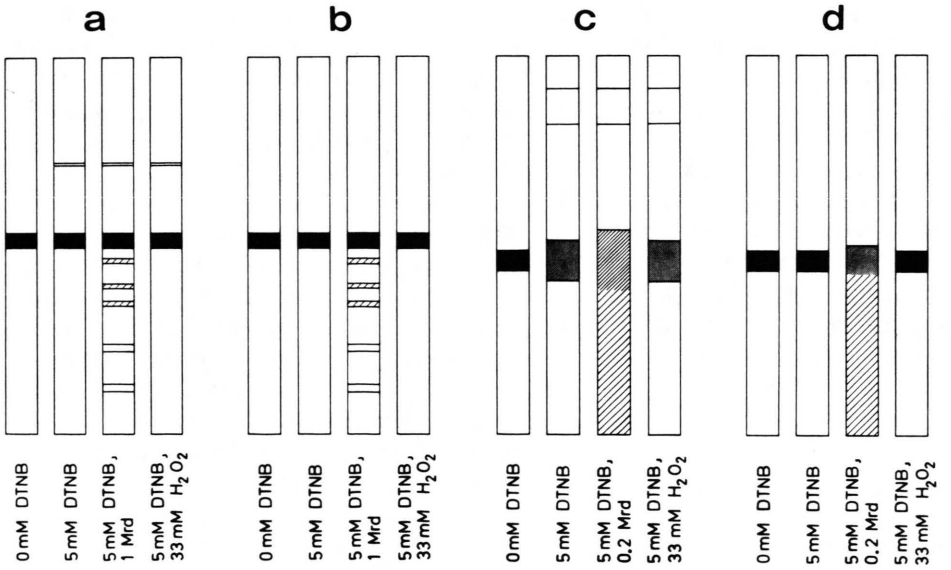

Fig. 8. PAGEs of unirradiated untreated, of DTNB-treated unirradiated, of DTNB-treated and X-irradiated, and of DTNB- and H_2O_2-treated unirradiated malate synthase in the presence of SDS and without 2-ME (a), in the presence of SDS and 2-ME (b), in the absence of SDS and without 2-ME (c), or in the absence of SDS but with $\leqq 10\%$ 2-ME (d). The enzyme samples ($c = 5$ mg/ml, 0.2 mM DTT) contained 0 or 5 mM DTNB and 0 or 33 mM H_2O_2; for X-irradiation doses of 1 or 0.2 Mrd were used. Incubation and electrophoreses were carried out as described for the X-irradiated or H_2O_2-treated enzyme respectively.

about 13 or 15% fragments have been found, for the SDS-PAGEs in the absence or presence of 2-ME respectively (Fig. 5e and f). Obviously DTNB reduced but did not prevent X-ray induced enzyme fragmentation, but it showed a significant protective effect against further subunit bonding.

PAGEs of the DTNB-treated and X-irradiated enzyme in the absence of SDS and in the absence of 2-ME show a weak band above the main band, but no band above the main band in the presence of 2-ME (Fig. 8c and d: 5 mM DTNB, 0.2 Mrd). This behaviour is in accord with a small amount of enzyme aggregates as a consequence of sulfhydryl-disulfide exchange reactions upon DTNB binding.

The electropherograms of the DTNB- and H_2O_2-treated unirradiated enzyme (Fig. 8a–d: 5 mM DTNB, 33 mM H_2O_2) gave nearly the same patterns as the DTNB-treated unirradiated enzyme, both in the presence or absence of SDS, and in the absence or presence of 2-ME during electrophoresis. The addition of H_2O_2 did not show any significant effect in the patterns, but led to a fading of the yellow-coloured enzyme-DTNB solution, indicating a reaction of H_2O_2 with the 2-nitro-5-thiobenzoate anion (*cf.* [22]). Subsequent addition of excess of 2-ME restored the yellow colour of the solution, probably by reaction of DTNB with 2-ME.

The behaviour of the DTNB-treated enzyme, *i.e.* absence of a distinct disulfide formation or the absence of a distinct enzyme aggregation upon X-irradiation or after H_2O_2 addition, may be explained by several reasons. The SDS-PAGEs suggest that the exposed sulfhydryls (which may be cross-linked by X-irradiation or H_2O_2 treatment in the absence of DTNB) were blocked by DTNB treatment; this may prevent disulfide bonding. The absence of distinct enzyme aggregates after having blocked the exposed sulfhydryls by DTNB suggests that these sulfhydryls are involved in any way in the aggregation of the enzyme.

Discussion and Conclusions

The chemical and electrophoretic investigations are influenced by various factors, such as use of a microlevel, polydispersity of the X-irradiated or H_2O_2-treated enyzme etc. (*cf.* Materials and Methods). Above all, a different age of the enzyme turned out to be responsible for a different stability of the enzyme: Enzyme freshly isolated appeared to be more stable against X-rays or H_2O_2 treatment than enzyme which had been stored for some time, *i.e.* with fresh enzyme higher X-ray doses or higher H_2O_2 concentrations were necessary to obtain the same electropherograms as for enzyme solutions stored for some time. A similar phenomenon is also well-known in biochemical work (*e.g.* effectiveness of proteolytic enzymes against aged proteins). Nevertheless the results obtained are able to deliver clear statements concerning the rationale underlying our presentation, *i.e.* the verification of the existence of aggregates, fragments, and unfolding of the enzyme or its subunits due to X-irradiation.

X-Irradiated enzyme

Though the quantitative results of the sulfhydryl determinations might be affected by various aspects (concentration of both protein and DTNB, irradiation conditions, formation of sulfenic acid etc.), they indicate a radiation damage of total sulfhydryl groups and simultaneously a partial unfolding of the enzyme by X-irradiation. Of course, no statements can be made on the immutability of the essential sulfhydryls by using only this technique.

The activity measurements, on the other hand (though they might also be influenced by various factors, *e.g.* age, initial specific activity and concentration of the enzyme), clearly show that X-irradiation leads to a distinct diminution of enzymic activity. The inactivation can be partially suppressed by the presence of DTT during X-irradiation (protective effect) or can be partially reversed afterwards by DTT (repair effect).

In this context it should be mentioned, that previous investigations on unirradiated enzyme indicated that the oxidation of the essential sulfhydryl groups (*e.g.* as a consequence of ageing) are responsible for changes both of enzymic activity and of a series of molecular parameters; some of these changes may be reversed also partially by excess of DTT ([5, 6, 35], detailed results to be published).

The electrophoreses performed in the presence or absence of the denaturant SDS convincingly demonstrated the occurrence of covalently connected subunits, of various definite fragments, of enzyme aggregates, and of a partial unfolding of the enzyme or its subunits.

The formation of covalent cross-links in X-irradiated malate synthase was clearly shown by the

SDS-PAGEs. The fact that the electropherograms obtained were well influenced by 2-ME (Figs. 2 and 3) clearly established the involvement of disulfide bridges. A similar conclusion may be drawn from the protective effect of DTT. Especially at higher X-ray doses, a considerable part of the covalent cross-links, however, must be attributed to links other than disulfides.

The PAGEs in the absence of SDS (Fig. 6) were influenced by the presence of DTT during X-irradiation, but DTT was not able to prevent enzyme aggregation. The fact that the presence of excess of 2-ME or DTT during electrophoresis showed no effect, suggests that the enzyme aggregates are not linked exclusively by disulfides. A similar conclusion can be drawn from preliminary SAXS studies which also showed that the addition of excess of DTT to X-irradiated malate synthase does not cleave the aggregates.

The PAGEs in the absence of SDS already showed at low X-ray doses bands due to enzyme aggregates, while the corresponding SDS-PAGEs did not show multiple bands (*cf.* Figs. 6a and 3a: 0.1 Mrd; Figs. 6b and 2b and 3b: 0.1 and 0.2 Mrd). This behaviour demonstrates the involvement of non-covalent bonds in enzyme aggregation.

The observed exclusion of the aggregates, formed upon X-irradiation at high X-ray doses (or upon addition of large amounts of H_2O_2), from the pores of the electrophoresis gel in the absence of SDS lends further support to the model of two-dimensional aggregation as proposed from our SAXS studies [10, 11, 13]. Particles formed by a two-dimensional aggregation are of much larger extension than compact three-dimensional aggregates of the same molecular weight would be.

The PAGEs obtained after having blocked sulfhydryls by DTNB demonstrated the absence of a distinct subunit cross-linking and of an enzyme aggregation upon X-irradiation. In connection with the PAGEs of samples X-irradiated in the absence of DTNB this suggests an involvement of sulfhydryls in enzyme aggregation. These sulfhydryls are not necessarily identical with the sulfhydryls available in the native state; sulfhydryls which become exposed as a consequence of X-irradiation may be blocked by excess of DTNB as well. The suppression of enzyme aggregation by DTNB was also confirmed by preliminary SAXS investigations (unpublished results). The involvement of sulfhydryls in enzyme aggregation is not necessarily a disulfide formation as follows from the comparison of the multiple bands in the absence of DTNB, in the presence or absence of SDS (*cf.* Figs. 2 and 3 with 6). Considering the role of DTNB, a possible scavenging effect of the aromatic disulfide DTNB must also be taken into account.

The difference in intensity of the multiple bands in the SDS-PAGEs of X-irradiated enzyme (1 Mrd) in the presence or absence of 2-ME (without 2-ME: 11% dimer, 6% trimer; with 2-ME: 8% dimer, 4% trimer) would correspond to a loss of 0.17 sulfhydryl groups per enzyme molecule. Indeed Table II shows a higher total sulfhydryl loss. This may be explained by an involvement of sulfhydryls in covalent bonds other than disulfides or in non-covalent bonds or by the formation of sulfenic acid or other oxidation products.

A recent SAXS study [37] on the X-ray induced aggregation of ribonuclease, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and serum albumin, in comparison with malate synthase, established that ribonuclease showed the highest rate of aggregation under the conditions of the experiment, though this enzyme does not possess free sulfhydryl groups.

The SDS-PAGEs do not allow to distinguish unambiguously between intra- and intermolecular covalent cross-linking. Nevertheless the fact that besides dimers of the subunit a considerable amount of trimers, but only a negligible amount of higher multiples, was found in the SDS-PAGEs may be regarded as a hint for intramolecular covalent cross-linking and also as a support for the suggested trimeric quaternary structure of the enzyme. Covalent intramolecular interchain bonds between the subunits may lead to a stabilization of the radiation damaged structure of the enzyme.

Examples for covalent as well as non-covalent cross-linking upon X-irradiation of aqueous solutions of proteins have been reviewed by Yamamoto [15]. A suppressive effect of oxygen on the covalent binding reaction has been reported [15]; it has also been suggested that hydrogen bonding between N-oxides and sulfoxides, produced by irradiation, and sulfhydryl groups can be performed. In this context it should be recapitulated that our experiments on malate synthase, which demonstrated the occurrence of covalent and non-covalent bonds between enzyme molecules and/or subunits, were carried out with

solutions containing oxygen [10–12]. In the case of lactate dehydrogenase, oxygen was reported to suppress both covalent and non-covalent aggregation of the protein molecules but to promote the formation of molecular fragments [19]. According to our SAXS studies [37], lactate dehydrogenase shows also a considerable amount of aggregates when X-irradiated in the presence of oxygen.

The occurrence of several bands in the SDS-PAGEs of X-irradiated enzyme, corresponding to molecular weights smaller than the subunit, clearly reflects the formation of definite fragmentation products, but does not allow statements whether peptide chain breaks in irradiated malate synthase lead to a disruption of the quaternary structure in the absence of denaturants. Such a disruption is possibly suggested by the observed decrease of the thickness radius of gyration of the enzyme upon prolonged irradiation (*cf.* [10, 11, 13]) and may be indicated by the electrophoretic patterns in the absence of denaturants. X-ray induced aggregates of enzymes may also consist of protein fragments; this was demonstrated by Schuessler *et al.* [38] for the aggregates of lactate dehydrogenase: gel filtration after incubation with SDS suggested that the protein fragments were held together by hydrophobic and electrostatic interactions.

The differences in the electrophoretic patterns of the enzyme X-irradiated in the absence or presence of 2 mM freshly dissolved DTT obviously reflect the protective effect of DTT against X-ray damage of malate synthase and its subunits. Similar protective effects were established by our SAXS and activity measurements of this enzyme (*cf.* [7, 10–12]; *cf.* also Table III) and by SAXS studies of other proteins [37]. But while the radioprotection against aggregation and inactivation is clear from these experiments, no protective effect could be found against fragmentation.

H_2O_2-Treated enzyme

The concentrations of H_2O_2 used in the PAGEs were higher than the H_2O_2 concentrations in the irradiated enzyme solutions (*cf.* Table I). The total amount of H_2O_2 formed during X-irradiation of aqueous enzyme solutions in our experiments may be estimated to about 1–2 mM H_2O_2. However, it seems plausible that H_2O_2 *in statu nascendi*, as produced by X-irradiation, is more effective than H_2O_2 during treatment.

The results obtained from sulfhydryl determinations and from activity measurements as well as the electrophoretic patterns obtained for the H_2O_2-treated unirradiated enzyme are very similar to those for the X-irradiated enzyme. The electropherograms also demonstrated the occurrence of covalent and non-covalent bonds, and of fragments at very high H_2O_2 concentrations. Part of the covalent bonds may also be attributed to disulfide bridges.

H_2O_2 is known to be a relatively non-specific oxidizing agent reacting with various amino acid residues (*cf.* [39]). The formation of sulfenic acid products (repairable *e.g.* by DTT) was reported to be the primary reaction of H_2O_2 oxidation of enzyme sulfhydryl groups [34, 23, 33].

As follows from the high yield of repair phenomena, it seems obvious that also in the case of malate synthase sulfenic acid products are formed. An inactivation to about 1/3 of initial activity, as observed upon treatment with 33 mM H_2O_2 (*cf.* Table III: 0.2 mM DTT), is in good accord with the loss of two sulfhydryl groups (*cf.* Table II: total sulfhydryls, determination in the presence of urea). The loss of one sulfhydryl group may be attributed to sulfenic acid formation as may be concluded from the restoration of enzymic activity to 2/3 of its initial value upon addition of DTT. The fact that the determination of sulfhydryls in the absence of urea yielded a higher sulfhydryl loss corroborates this assumption.

A possible role of H_2O_2 in the X-ray damage of the enzyme

The similarity of the electrophoretic patterns of X-irradiated or H_2O_2-treated malate synthase suggests that besides other oxidants ($OH^{\cdot}$, $HO_2^{\cdot}$, O_2^{-}) also H_2O_2 might play a role in the X-ray induced aggregation of the enzyme.

Similar conclusions concerning a possible involvement of H_2O_2 in the X-ray induced inactivation of the enzyme may be drawn from the results of the enzymic tests: The repair effect initiated by DTT indicates the formation of sulfenic acid products upon X-irradiation (*cf.* Table III: 1 Mrd). The effect was more pronounced when irradiation was performed in the presence of 2 mM DTT; DTT was shown to favour a higher H_2O_2 concentration upon X-irradiation. In the case of the sulfhydryl enzymes

papain and glyceraldehyde-3-phosphate dehydrogenase the formation of sulfenic acid products upon irradiation was proven to be due to oxidation by H_2O_2 [33, 40].

In view of these findings an involvement of H_2O_2 in the X-ray damage of the sulfhydryl enzyme malate synthase may be expected. However to make final statements on the role of H_2O_2 and of other radiolysis products further investigations have to be carried out. Similar investigations as with the substrate-free enzyme have also to be performed with the enzyme-substrate complexes in order to get further insight into the different protective effects of the substrates against radiation damage.

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